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   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

   7455603001

4. Title of the invention — HEPARANASE ASSAY

5. Name of your agent *(if you have one)* — Marks & Clerk

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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DUPLICATE

HEPARANASE ASSAY

The present invention relates to an assay for heparanase activity

Endoglycosidases are capable of cleaving polymers comprised of glycosaminoglycan units (so called "GAG polymers"). Enzymes that cleave a GAG polymer internally are known as endoglycosidases and include heparanase which cleaves heparan sulphate GAG, hyaluronidase which cleaves hyaluronic acid GAG and chondroitinase which cleaves chondroitin sulphate GAG.

Heparanase has been described in T&B lymphocytes, monocytes, neutrophils, platelets, endothelial cells and in liver and placental tissue. This endoglycosidase activity which degrades heparan sulphate GAG has been variously called heparanase, heparinase, heparitinase and heparan sulphate lyase and almost certainly represents a family of enzymes with different specificities for the internal cleavage site in the GAG polymer.

Heparan sulphate glycosaminoglycans (HSGAGS) of which heparin, the anticoagulant is an example, are highly N- and O-sulphated carbohydrate polymers of glucuronic (sometimes iduronic) acid and glucosamine (sometimes N-acetylated). The high anionic charge is not randomly distributed throughout the polymer but is thought to form clusters of charged residues imparting specific functional binding properties to the sequence. This is demonstrated by the most important anti-coagulant properties of the pentasaccharide sequence in heparin and its interaction with anti-thrombin III..

HSGAG is present in the extracellular matrix, in basement membranes and on cell surfaces. HSGAG is known to play an important role in linking the component structural proteins of the basement membrane, controlling the permeability properties of the membrane (best illustrated in the glomerular basement membrane) and controlling the bio-availability of cytokines and growth factors.

Heparanases are known to play important roles in areas of discovery biology

a) cancer metastasis; the most metastatic cells display the highest levels of heparanase enzyme which may enable cancer cells to degrade HSGAG in ECM and basement membranes to facilitate tissue invasion. Cancer cells secreting heparanase may support angiogenesis, tumour vascularisation and therefore tumour growth. This is a prime area for developing novel antagonists to heparanase as part of a therapeutic strategy to supress and inhibit metastasis.

b) in inflammatory joint disease e.g. rheumatoid arthritis; angiogenesis in the joint may support the chronic inflammatory pathology. Agents inhibiting heparanase in the joint may reduce angiogenesis and therefore suppress the formation of the inflammatory pannus of tissue which causes degradation of cartilage and erosion of bone.

c) in proteinuric disease; HSGAG expression within the glomerular basement membrane controls the passage of anionic charged proteins e.g. albumin. In early diabetic nephropathy there is evidence of reduced basement membrane HSGAG associated with microalbuminuria. Furthermore, a number of clinical and experimental studies document reduction in proteinuria associated with low molecular weight heparin treatment. These findings support the role of increased heparanase expression within the glomerulus which might be treated with suitable antagonists.

Known assays (summarised below) for heparanase activity have a number of disadvantages.

(i) Radiolabelled substrate.

In vitro metabolic radiolabelling of cell cultures with $^{35}SO_4$ or ^{3}H incorporated into HSGAG is commonly used for detecting heparanase. The radiolabelled GAG

may be used following isolation and purification by coupling to a solid phase support. Alternatively, radiolabelled ECM laid down on culture dishes with the cell layer removed, may be used as substrate. Alternatively, ^{3}H or ^{125}I labelling of purified HSGAG or heparin is performed and the labelled substrate coupled to a solid phase. Heparanase activity is detected by loss of radiolabel and or reduction in molecular size of labelled species.

The radiolabelling procedures are hazardous and require special disposal procedures. Large variations in specific activity of substrate occur between batches. The batches of $^{35}SO_4$ substrate have a short lifetime of 2 to 3 months.

(ii) HS Substrate showing sensitivity to other enzymes

Assays claiming to measure heparanase that use heparan sulphate proteoglycan as the substrate and detect apparent enzyme activity by identifying release of labelled HSGAG or a reduction in the size profile of HSGAG (using isotope or other label) by molecular sieving are not specific for heparanase. A wide range of proteases present in the sample could act on the proteoglycan component to solubilise significant quantities of HS chains and cause reduction in size by further removal of attached protein. Other enzymes e.g. hyaluronidase may cleave the substrate. Few current assays have demonstrated insensitivity to this range of enzymes.

(iii) Sensitivity and quantitation

Assays requiring long incubation times (4-24 hours) to achieve an effect indicate a lack of sensitivity and leave open the possibility of interference by non-specific factors (e.g. proteases).

Assays requiring molecular sieving to identify the outcome of heparanase activity are only qualitative in nature

(iv) Multiple sample handling

Assays requiring centrifugation or molecular sieving to separate the products of heparanase activity only allow small batch assays to be performed.

It is an object of the present invention to obviate or mitigate the above mentioned disadvantages.

According to a first aspect of the present invention there is provided a method of assaying a sample to determine heparanase activity thereof the method comprising

(i) incubating the sample in the presence of a first solid phase support having immobilised thereon an HSGAG polymer substrate for the heparanase, the said substrate being insensitive to the action of proteases and the said substrate having bonded thereto a first binding moiety and having further bonded thereto a paracrine cell regulator capable of binding to HSGAG,,

(ii) treating the incubated sample with a second solid phase support having a second moiety provided thereon capable of immobilising HSGAG polymer substrate cleaved from the first solid phase support on said second solid phase support by binding with the paracrine cell regulator or the first moiety,

(iii) generating a measurable signal from cleaved substrate immobilised in the second support solid phase, and

(iv) measuring the signal on the second solid phase support which has been separated from the first solid phase support.

In a second aspect, the invention provides, for the use in an assay as defined above, a solid phase substrate (equivalent to the aforementioned first substrate) having

immobilised thereon an HSGAG polymer substrate for heparanase, said substrate having bonded thereto a first binding moiety and having further bonded thereto a paracrine cell regulator capable of binding to HSGAG.

In a third aspect, the invention provides the combination of first solid phase support as defined in the previous paragraph and second solid phase support having provided thereon a second moiety capable of binding to either the first moiety or the paracrine cell regulator to form a complex therewith.

In a fourth aspect, the invention provides an assay kit comprising the combination of the previous paragraph and signal developing agents to generate a signal representative of the result of the assay.

The paracrine cell regulator may, for example, be a growth factor, cytokine or chemokine.

The assay of the invention allows rapid quantitation of heparanase activity in a sample having, or potentially containing, such activity.

The invention is further described with reference to the paracrine cell regulator being an HSGAG binding growth factor but it is to be understood that the invention is equally applicable to other paracrine cell regulators.

The assay is conducted using a solid phase format. Step (i) of the assay uses a first solid phase support on which is immobilised an HSGAG polymer substrate which is cleavable by the heparanase present (or potentially present) in the sample. Bonded to this polymer substrate is

(a) HSGAG binding growth factor, and

(b) a first binding moiety

Given that there is heparanase activity in the sample, the procedure of step (i) results in cleavage of HSGAG polymer substrate in an amount which is dependent on the degree of herparanase activity in the sample and its ability to cleave the HSGAG polymer substrate in the presence of the growth factor bonded thereto.

Any cleaved HSGAG polymer substrate (having both the first moiety and the growth factor bonded thereto) is released into the analyte phase. Confirmation that HSGAG-growth factor has been removed from the solid phase in step (i) can be obtained after step (ii) by incubating the washed heparanase treated solid phase with (strept)avidin-conjugated enzyme or anti-growth factor conjugated enzyme and comparing the signal with that obtained from a control solid phase. Heparanase activity significantly reduces the signal obtained.

In step (ii), the analyte phase is treated with a second solid phase support system incorporating a second moiety capable either of forming a complex with the first binding moiety or capable or forming a complex with the growth factor. As a result, complex formation between the first and second moieties occurs or complex formation between the growth factor and the second moiety occurs resulting in the cleaved fragments becoming immobilised on the second solid phase support. Either the first moiety or the growth factor remains unbound and is used for signal development.

In step (iii), a measurable signal is generated from the cleaved fragments immobilised on the second solid phase support. The signal may be dependent on the growth factor component of the complex (where the complex is captured by the first binding moiety and detected with anti-growth factor antibody) and enzyme coupled anti-species antibody. Alternatively, where the complex is captured by anti-growth factor antibody the signal may be generated by an enzyme coupled to a third moiety capable of binding to the first moiety. The signal may for example, be a colorimetric signal and be measured by a spectrophotometer.

Finally in step (iv), the signal generated in step (iii) is measured. This measurement is effected with the second solid phase support system separated from the first such system (i.e. that employed in step (i)). Such separation is preferably effected between steps (ii) and (iii).

The measured signal is representative of the specific heparanase activity in the original sample. More particularly, the measured signal directly reflects the biological action in generating complexes of growth factor-HSGAG that would be produced in physiological and pathological states.

A critical control point in these pathologies is the bioavailibility of important cytokine, chemokine and growth factors. On release from the secreting cell, most of the mediator(>70%) would normally interact with HSGAG in extracellular matrix (ECM) to form a stable reservoir of mediator. Only a fraction of the secreted mediator directly and immediately binds to the specific mediator receptor on the target cell to initiate cell signalling. Heparanase enzymes provide critical control of this reservoir by releasing these biologically specific growth factors from ECM that can bind their target receptors and initiate signalling.

Cytokines that bind HSGAG include IL-1α and IL-1β, IL-2, IL-4, IL-7, IL-8, IL-12, TNFα, IFNα. Chemokines that bind HSGAG include IP-10, MCP-1. Growth factors that bind HSGAG include VEGF, FGF-1, FGF-2, TGFβ1, PDGF, HGF. The term growth factor will be used to denote any of these mediators. The invention will measure heparanase activity using any of these heparan sulphate binding GFs

The heparanase-released growth factor in the form of a complex of variable length of HSGAG sequence bound to growth factor may be significantly more active biologically than the native growth factor.

The binding sites in HSGAG that interact with these growth factors are poorly described at present and it may be that each growth factor has a unique binding site. Alternatively, some growth factors may share a certain binding sequence in HSGAG.

Heparanases from different cell sources may be differentiated from each other by their ability to solubilise particular growth factors or combinations of growth factors from HSGAG. This will happen where the binding of growth factor to the HSGAG substrate protects or sterically occludes a potential enzyme cleavage site. Thus by use of a panel of growth factors bound to HSGAG, it may be possible to attribute a particular pattern of GF solubilisation to a heparanase from platelets compared to synovial fluid or tumour. This may be of importance for instance in differentiating the action of a tumour heparanase from platelet derived heparanase.

Of considerable importance in the design of antagonists of heparanase function is the use of this method to generate selective heparanase inhibitors of exquisite specificity such that inhibition is confined to those HSGAG cleavage sites linked with solubilisation of a certain growth factor. Thus, for instance, a specific inhibitor of VEGF solubilisation may reduce and suppress tumour angiogenesis and metastasis without inhibiting the immune functions of a platelet heparanase in solubilising IFNα to protect from infection during wound healing.

The assay is unaffected by the presence of proteases, hyaluronidase and chondroitinases. The assay is sensitive only requiring incubation times at step (i) of 30-60 minutes.

The method of the invention may be used for determining heparanase activity in a biological sample, e.g. for the purposes of diagnosing or characterising a particular medical condition. It is however envisaged that an important application of the invention will be in the screening of potential inhibitors or enhancers of heparanase activity. Thus, for example, the assay may be conducted with an analyte containing an heparanase and a putative inhibitor therefore to determine the effect (or otherwise) of that inhibitor.

Description of Preferred Embodiments

The assay of the invention is particularly useful for determining heparanase activity, e.g. for the purpose of evaluating potential inhibitors thereof.

The GAG polymer substrate immobilised on the support is preferably heparan sulphate glycosaminoglycan.

The first solid phase support system (i.e. the system in which the HSGAG polymer substrate is immobilised) is preferably a microtitre well. The assay may be conducted using a tray of microtitre wells or larger arrays having immobilised HSGAG polymer substrate. As such, the assay of the invention may be effected using robotic technology to screen compounds for anti-heparanase activity. When using a microtitre well as the first support system, the second solid phase support system may be a microtitre plate lid with a coated probe extending into each assay well as the second support system, for example, NUNC TSP screening system or FALCON FAST system. In this case, step (i) and step (ii) of the assay take place during the same incubation time. Alternatively, the second solid phase may be another coated microtitre well and step (i) and step (ii) occur sequentially and require physical transfer of the contents of the first well to the second well.

If the cleaved substrate is to be captured (on the second solid phase support) by the first moiety then the first moiety may be biotin and the second moiety strept(avidin). In this case the signal may be generated by means of an anti-growth factor antibody and enzyme coupled anti-species antibody.

If the cleaved substrate is to be captured by the growth factor then the second moiety may be anti-growth factor antibody. In this case the first moiety may be biotin and the signal generated by an enzyme coupled to (strept)advin (as the aforementioned third moiety).

The method of the invention may be effected using the following protocol.

The GAG polymer substrate may be tagged with biotin using simple photobiotylation chemistry. A mixture of photobiotin (1mg/ml) and HSGAG (10mg/ml) in phosphate buffered saline (PBS) at a ratio of 2:1 (vol/vol) is exposed to UV light emitted from a Mercury arc HBO50 W lamp used as a microscope UV source for 21 minutes at a distance of 10 cm. The biotinylated HSGAG is dialysed against PBS for 18 hours at 4°C and stored at –20°C.

The immobilisation of the GAG polymer substrate on the solid phase support is carried out after the tagging with the first complex forming moiety and may be effected by oxidising the substrate with periodate and incubation with carbohydrate binding multiwell plates (for example, COSTAR or NUNC plates may be used) to yield a surface to which the substrate is covalently and stably bonded. Specifically, sodium periodate at 10μM and biotinylated HSGAG at 5μg/ml in sodium acetate buffer (10mM, pH 4.0) are allowed to interact for upto 15 minutes and then 100μl of the activated biotinylated HSGAG is added to each well of the carbohydrate binding assay plate for 1 hour, shaking at 37^{++}C. The plates are then washed thoroughly in PBS containing 0.1% Tween 20. 100μl of 1% bovine serum albumin (BSA) in PBS is added to each well and incubated at 37°C for 60 minutes to block any nonspecific binding sites on the plate. Following washing with PBS, the coated plate may be used immediately or stored wrapped in cling film at 4°C for several months.

Once the GAG polymer substrate has been immobilised in the first support, it (i.e. the substrate) may be treated with a GAG binding growth factor (e.g. VEGF, TGF β1, MCP-1, IFN-γ, see list in claim 8). 100μl of GF at 1-5μg/ml in PBS is added to each well and incubated for 18 hours at 4°C. Following washing in PBS, the plate is ready for use.

The second solid phase support may be prepared by coating the support material with the second complex forming moiety (e.g. (strep)avidin blocked with BSA). The microwell lid probes or wells, where the second solid phase is another

well, are coated with (strept)avidin at 1-5μg/ml in PBS for 18 hours and following washing in PBS they are blocked with BSA as above.

To carry out the assay, the analyte having (or potentially having) heparanase activity is incubated in the presence of the first solid phase support system. The analyte is diluted in enzyme assay buffer which is sodium acetate (0.1M, pH 5.5 containing Ca^{2+} (5mM), Cu^{2+} (1mM) and Fe^{3+} (1mM). Each assay includes as controls, wells without biotinylated HSGAG coating and wells which do not receive the test sample. The heparanase assay is allowed to proceed for 1 hour normally (but may range from 30minutes to 4 hours) on a plate shaker at 37°C. Where the second solid phase is a well lid coated probe, steps (i) and (ii) occur simultaneously, where the second solid phase is another coated well, the supernatant from the first well is transferred to the second well and incubated for 2 to 24 hours.

As a result, any biotinylated HSGAG-GF substrate cleaved from the first solid phase support during incubation becomes immobilised on the second support which may now be separated from the analyte.

A signal is then developed. Where the second solid phase used strep(avidin) to capture the complex through its biotin moiety, this may be achieved by incubating the second solid phase support with anti-growth factor antibody followed by enzyme-conjugated anti-species IgG. Where the second solid phase used anti-growth factor antibody to capture the complex, signal may be developed by incubating with enzyme-conjugated strep(avidin). Confirmation that the substrate bound to the first solid phase has been cleaved can be obtained by incubation with either enzyme conjugated strep(avidin) or enzyme conjugated antibody. The preferred enzyme conjugated to these detection reagents is peroxidase and therefore the amount of peroxidase activity associated with the well probe may be revealed using a standard TMB substrate colour change and spectrophotometry.

CLAIMS

1. A method of assaying a sample to determine heparanase activity thereof the method comprising

(i) incubating the sample in the presence of a first solid phase support having immobilised thereon an HSGAG polymer substrate for the heparanase, the said substrate being insensitive to the action of proteases and the said substrate having bonded thereto a first binding moiety and having further bonded thereto a paracrine cell regulator capable of binding to HSGAG,,

(ii) treating the incubated sample with a second solid phase support having a second moiety provided thereon capable of immobilising HSGAG polymer substrate cleaved from the first solid phase support on said second solid phase support by binding with the paracrine cell regulator or the first moiety,

(iii) generating a measurable signal from cleaved substrate immobilised in the second support solid phase, and

(iv) measuring the signal on the second solid phase support which has been separated from the first solid phase support.

2. A method as claimed in claim 1 wherein the HSGAG polymer substrate is covalently bonded to the first solid phase support system.

3. A method as claimed in claim 1 or 2 wherein the first solid phase support system is a microtitre well.

4. A method as claimed in claim 3 wherein the second solid phase support system is a plate lid probe.

5. A method as claimed in claim 3 wherein the second solid phase support system is a microtitre well.

6. A method as claimed in any of claims 1 to 5 wherein the second solid phase support system is separated from the first solid phase support system between steps (ii) and (iii).

7. A method as claimed in any one of claims 1 to 6 wherein, in step (ii), HSGAG polymer substrate cleaved from the first solid phase support is immobilised in the second solid phase support by binding of the first and second moieties.

8. A method as claimed in claim 7 wherein the first moiety is biotin and the second moiety is strept(advin).

9. A method as claimed in claim 7 or 8 wherein the signal is generated by an anti- paracrine cell regulator antibody and enzyme coupled anti-species antibody.

10. A method as claimed in claim 9 wherein the signal is developed by incubating the second solid phase support with anti- paracrine cell regulator antibody followed by peroxidase anti-species IgG.

11. A method as claimed in any one of claims 1 to 6 wherein, in step (ii), the HSGAG polymer substrate cleaved from the first solid phase support is immobilised on the second solid phase support by binding of the paracrine cell regulator and the first moiety.

12. A method as claimed in claim 11 wherein the signal is generated by an enzyme coupled to a third moiety capable of binding to the first moiety.

13. A method as claimed in any one of claims 1 to 12 wherein the paracrine cell regulator is a growth factor, a cytokine or a chemokine.

14. A method as claimed in claim 13 wherein the paracrine cell regulator is a growth factor and is selected from VEGF, TGFβ1, FGF-1, FGF-2, HGF, PDGF, IL-1α, IL-1β, IL-2, IL-4, IL-7, IL-8, IL-12, IP-10, MCP-1, TNFα or IFN-α.

15. A method as claimed in any of claims 1 to 14 for determining the activity of a putative inhibitor of heparanase activity.

16. A solid phase substrate having immobilised thereon an HSGAG polymer substrate having bonded thereto a first moiety capable of binding to a second moiety and a paracrine cell regulator capable of binding to HSGAG, said first moiety or paracrine cell regulator being capable of binding to a second moiety.

17. A substrate as claimed in claim 16 wherein the paracrine cell regulator is a growth factor, chemokine or cytokine.

18. The combination of a solid phase support as claimed in claim 16 or 17 and a second solid phase support having provided therein said second moiety.

19. An assay kit comprising the combination as claimed in claim 18 and signal developing agents to generate a signal representative of the result of the assay.

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